IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant	:	Wright, J.L.C.
Appln. No.	:	09/385,834
Filed:	:	August 30, 1999
Title	:	A Nutritional Supplement for Lowering Serum Triglyceride and Cholesterol Level
Grp./A.U.	:	1616
Examiner	:	S. N. Qazi
Docket No.	:	76891

DECLARATION PURSUANT TO 37 CFR § 1.132

I, H. STEPHEN EWART Ph.D., hereby declare that:

1. I am currently employed by Ocean Nutrition Canada Limited, the assignee of the above-identified application, in the capacity of Principal Research Scientist. I am currently responsible for the ongoing research project from which the above-identified patent application arose. Details of my education, employment in research, and my academic publications are set forth in Exhibit 1 hereto. In view of my education and my involvement with the research project relating to this application, I have extensive background and experience in the area of nutritional supplements for lowering triglyceride and cholesterol levels.

2. I have carefully reviewed this patent application, the Office Action mailed April 26, 2002, and the references cited therein.

3. Claims 1, 5-11, 34 and 39 of the instant application stand rejected as being obvious over U.S. Patent No. 5,770,749 to Kutney *et al.* and U.S. Patent No. 4,879,312 to Kamarei *et al.* The Examiner states that Kutney *et al.* teach that phytosterols are effective in lowering plasma cholesterol levels and that Kamarei *et al.* teach that a diet rich in omega-3 fatty acids has beneficial effects in humans, including a reduction in plasma cholesterol and triglyceride levels. The Examiner goes on to state that the present claims differ from the references in claiming a nutritional supplement by employing a combinati n of phytosterols and an omega-3 fatty acid. The Examiner therefore concludes that it would be obvious to one skilled

in the art at the time of the invention to employ phytosterols in combination with omega-3 fatty acids in compositions and methods for lowering cholesterol and triglycerides in the bloodstream of a subject, because these agents are known individually for the treatment of the same disorder.

4. It is very important to recognize that the present invention concerns not mere mixtures of sterols and omega-3 fatty acids but rather *esters* of a sterol and an omega-3 fatty acid. For the purposes of making nutritional supplements, there are very important differences between a mixture of a sterol and a fatty acid, and a composition wherein the sterol has been chemically joined to the fatty acid through ester linkage.

5. Mere mixtures of sterols and omega-3 fatty acids are not useful for making nutritional supplements at least in part because the free sterol does not dissolve well into the fatty acid. When mixed together, the sterol remains crystalline, and the particles merely become suspended in the omega-3 fatty acid oil, resulting in a gritty paste-like material. This paste cannot be added to food products without substantially degrading their aesthetic properties. For example, a mere pasty mixture of a sterol and a fatty acid could not be added to margarine, without unacceptably altering the appearance, texture, and flavour of the margarine. Similarly, a pasty mixture is difficult to microencapsulate, the preferred means of delivering the nutritional supplement in cake mixes, baked goods, ice cream, etc.

6. Such pastes are also very difficult to formulate into pharmaceutical compositions. For instance, the paste cannot be easily packaged in a capsule, which is the preferred single dosage format. Similarly, the pasty composition could not be packaged in a liquid form, which requires a homogenous oil.

7. The present invention overcomes the above-identified problems by chemically joining the sterol to the omega-3 fatty acid through an ester linkage. The sterol ester thereby produced is an oily, viscous liquid, suitable for introduction into food products and for packaging in capsules and the like. Three to four grams of this sterol ester can be dissolved in about 20 grams of margarine or other dietary fat source, without altering significantly the texture/taste profile of the product.

8. As discussed in detail in my Declaration filed on April 12, 2001, the sterol esters of omega-3 fatty acids of the invention are effective for lowering *both* cholesterol and triglyceride levels in the blood of animals. This is, in fact, a very surprising result due to the differing mechanisms of action of sterols and omega-3 fatty acids.

9. Phytosterols are not absorbed in the digestive tract to any great extent. This is acknowledged in the Kutney *et al.* reference at column 2, lines 6-7 where it is stated that phytosterols have no nutritional value to humans, i.e., the phytosterol does not get absorbed into the bloodstream. The mechanism by which phytosterol lowers blood cholesterol appears to involve inhibition of cholesterol absorption in the small intestine by competing with cholesterol at critical points in the uptake process.

10. In contrast, in order to effect a reduction in bloodstream *triglyceride* levels, omega-3 fatty acids must be absorbed from the intestinal lumen into the bloodstream. Fish oil omega-3 fatty acids must travel in the bloodstream to the liver where they modulate the activity of several enzymes of carbohydrate and lipid. The overall effect is the promotion of hepatic fatty acid oxidation and reduction of triacylglycerol synthesis, with a consequent reduction of triacylglyverol release into the circulation (see article cited in current patent application: Connor and Connor ,1997, Are fish oils beneficial in the prevention and treatment of coronary artery disease? Am. J. Clin. Nutr. 66 (suppl.): 1020S-1031S .).

11. Therefore, at the time of the invention, it was unknown whether this opposing requirement would be met. In particular, would the sterol component of the ester prevent the fatty acid from being absorbed into the bloodstream? While there are digestive enzymes in the intestinal lumen with esterase activity that could potentially free the fatty acid from its ester linkage with the sterol, the degree to which this would occur was unpredictable, and therefore, it was unclear whether sufficient of the omega-3 fatty acids would be released to have a significant impact on serum triglyceride levels.

12. Moreover, contrary to what is stated in Kamarei *et al.*, the preponderance of scientific evidence is that omega-3 fatty acids do not lower

cholesterol, and may actually increase it. Harris (1989) J. Lipid. Res. 30:785-807, discussed in the present patent application at page 7, lines 25-27, concluded that fish oil consumption (omega-3 fatty acids) results either in no change in serum cholesterol, or actually leads to an increase in LDL cholesterol. Similarly, a recently reported study found that EPA and DHA, the principal omega-3 fatty acids found in fish oil, *increased* LDL cholesterol levels (see Stalenhoef *et al.* (2000) The effect of concentrated N-3 fatty acids versus gemfibrozill on plasma lipoproteins, low density lipoprotein heterogeneity and oxidizability in patients with hypertriglyceridemia. Atherosclerosis 153:129-138, attached hereto as Exhibit 2).

13. LDL cholesterol is the form of blood cholesterol lowered by ingestion of sterols. Thus, irrespective of the above-described complications arising from the esterification of sterols with the omega-3 fatty acids, based on the totality of the available scientific literature, it would have been expected that the cholesterol-*increasing* effect of the omega-3 fatty acid might reduce or counteract the cholesterol-lowering effect of the sterol. It would not have been expected that the combination of the sterol and the omega-3 fatty acid, particularly in esterified form, would result in a reduction in both cholesterol and triglyceride levels, as disclosed in the present application.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

H. Stephen Ewart, Ph.D.

July 10/2002
Date

EXHIBIT 1

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Education

09/87 - 09/93	Ph.D. in Biochemistry, Memorial University of Newfoundland St. John's, Newfoundland Supervisor: Dr. J.T. Brosnan
09/84 - 05/86	M.Sc. in Biology, Mount Allison University Sackville, New Brunswick Supervisor: Dr. W.R. Driedzic
09/79 - 04/83	B.Sc. (Honours) in Biology, Mount Allison University Sackville, New Brunswick

Employment in Research

04/01 – present	Principal Research Scientist Ocean Nutrition Canada Ltd. Halifax, NS
04/99 – 04/01	Senior Research Scientist Ocean Nutrition Canada Ltd. Halifax, NS
04/96 – 03/99	Postdoctoral fellow, Department of Pharmacology & Therapeutics University of Calgary Calgary, Alberta
10/93 - 03/96	Postdoctoral fellow, Division of Cell Biology Hospital for Sick Children Toronto, Ontario
05/86 - 07/87	Research assistant, Department of Bi logy Mount Allison University Sackville, New Brunswick

Honours and Awards

09/93 - 09/95 Hugh Sellers Postdoctoral Fellowship
Banting and Best Diabetes Centre

1992 Merck Frosst - Canadian Biochemical Society Student Travel Award
01/88 - 01/91 Memorial University Graduate Student Fellowship
Memorial University of Newfoundland

09/80 - 05/83 Wilkinson Scholarship
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09/79 - 05/80 Entrance Scholarship
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Teaching experience

09/87 - 12/92 Laboratory teaching assistant for biology and biochemistry courses at Memorial University
Introductory Biochemistry, 3100 (6 semesters)
Techniques in Biochemistry, 4211 (3 semesters)
Introductory Biology (1 semester)
Pharmacy Tutorial (1 semester)

09/84 - 05/86 Laboratory teaching assistant for biology courses at Mount Allison University
Animal Physiology, 3210 (2 semesters)
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Analytical Chemistry (1 semester)
Introductory Biology and Ecology (3 semesters)

Publications

Refereed papers

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Professional Memberships

Canadian Institute of Food Science and Technology

American Diabetes Association

American Heart Association

Nova Scotia Institute of Science (Councillor)

Exhibit 2

ELSEVIER

Atherosclerosis 153 (2000) 129–138

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The effect of concentrated n-3 fatty acids versus gemfibrozil on plasma lipoproteins, low density lipoprotein heterogeneity and oxidizability in patients with hypertrygliceridemia

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Abstract

We evaluated in a double-blind randomized trial with a double-dummy design in 28 patients with primary hypertriglyceridemia, the effect of gemfibrozil (1200 mg/day) versus Omacor (4 g/day), a drug containing the n-3 fatty acids eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), on lipid and lipoprotein levels, low density lipoprotein (LDL) subfraction profile and LDL oxidizability. Both Omacor and gemfibrozil therapy resulted in a similar significant decrease in serum triglyceride (TG), very low density lipoprotein (VLDL) triglyceride and VLDL cholesterol concentrations and an increase in high density lipoprotein (HDL) and LDL cholesterol concentrations. The increase in LDL cholesterol was due to a significant increase in cholesterol content of the relatively buoyant LDL subfractions LDL1, LDL2 and LDL3, whereas the relative contribution of the dense LDL subfractions LDL4 and LDL5 to total LDL tended to decrease. So, both therapies resulted in a more buoyant LDL subfraction profile, reflected by a significant increase of the value of parameter K (+10.3% on Omacor vs +26.5% on gemfibrozil therapy, gemfibrozil vs Omacor $P > 0.05$). Cu^{2+}-induced oxidation of LDL was measured by continuous monitoring of conjugated dienes. After 12 weeks of Omacor treatment LDL appeared more prone to oxidative modification in vitro than LDL after gemfibrozil treatment, as measured by the significantly decreased lag time, preceding the onset of the lipid peroxidation. In both groups the rate of oxidation did not change with therapy. The amount of dienes formed during oxidation increased significantly on Omacor treatment, but not on gemfibrozil treatment. Plasma thiobarbituric acid reactive substances were higher after Omacor and lower after gemfibrozil treatment, although not significantly. We conclude that both Omacor and gemfibrozil have favorable effects on lipid and lipoprotein concentrations and the LDL subfraction profile. However, Omacor increased the susceptibility of LDL to oxidation, whereas gemfibrozil did not affect the resistance of LDL to oxidative modification in vitro. The clinical relevance of these changes remains to be established in the light of other postulated favorable effects of n-3 fatty acids on the course of cardiovascular disease.

Keywords: Fish oil; Gemfibrozil; Omega-3 fatty acids; Hypertriglyceridemia; Low density lipoprotein subfractions; Low density lipoprotein oxidation

1. Introduction

Subjects with moderate hypertriglyceridemia are considered to be at increased risk for coronary heart disease (CHD), especially men over age 50 with low high density lipoprotein (HDL) cholesterol levels [1]. Several potential mechanisms have been suggested to contribute to this phenomenon, including an enhanced atherogenic potential of l w density lipoprotein (LDL) in the hypertriglyceridemic subjects [2–4]. LDL isolated from hypertriglyceridemic subjects is polydisperse

Abbreviations: CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; FA, fatty acids; FCH, familial combined hyperlipidemia; HDL, high density lipoprotein; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids; TG, triglycerides; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein.

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defined by the presence of multiple LDL subfractions over a broad density range, with the mean LDL subfractions being abnormally small and dense [4,5]. This dense LDL subfraction profile has been associated with an increased risk of CHD [6–8]. In addition, LDL isolated from hypertriglyceridemic subjects is more prone to in vitro oxidative modification than LDL from normotriglyceridemic subjects [4]. The oxidative modification of LDL has been implicated in the initiation and progression of atherosclerosis [9]. So, LDL in hypertriglyceridemic subjects is characterized by a dense LDL subfraction profile and an enhanced susceptibility to oxidation, both contributing to an enhanced atherogenic potential of LDL and thus increased risk of atherosclerosis.

Because of the reported increased risk for premature atherosclerosis, treatment with lipid-lowering drugs is frequently indicated. Both marine n-3 fatty acids (FA) and fibrates are very potent hypotriglyceridemic agents; however, both can also raise LDL cholesterol concentrations, especially in hypertriglyceridemic subjects [10–13]. Only a few studies are available that address the effect of n-3 FA [14–16] and fibrates [4,17] on LDL heterogeneity. Furthermore, dietary n-3 FA are incorporated into lipoproteins, thereby potentially affecting the susceptibility of LDL to oxidative modification. There are conflicting results, however, between studies on the effects of n-3 fatty acid supplementation on LDL oxidizability [16,18–23], whereas only few studies report the effect of fibrates on this parameter [4,17,24].

The present study was undertaken to compare directly the effects of concentrated n-3 FA (Omacor®) vs gemfibrozil on LDL heterogeneity and LDL oxidizability in hypertriglyceridemic patients.

2. Methods

2.1. Patients

A total of 30 patients with primary hypertriglyceridemia (triglyceride (TG) levels between 4.0 and 28.0 mmol/l), confirmed by repeated measurements, were recruited from the outpatient lipid clinic of Nijmegen University Hospital (18 patients) and Amsterdam Academic Medical Centre (12 patients). Exclusion criteria were secondary causes for dyslipidemia, including a history of diabetes mellitus, or apolipoprotein phenotype E2/E2. The participants continued their standard lipid-lowering diet throughout the trial (American Heart Association Step I diet: <30% of total calories/day from fat (maximum 10% saturated fat) and cholester l <300 mg/day). Other concomitant medication was maintained unchanged during the study. None of the subjects used vitamin supplements, antioxidants or oral blood glucose low ring agents. The protocol was approved by the ethical committee of our institution and written informed consent was obtained from all subjects.

2.2. Study design

This study was a double-blind trial with a double-dummy design. At the start of the study lipid-lowering medication was stopped (week −6), followed by a wash-out period of 4 weeks (week −6 to −2). Baseline plasma lipid values were measured twice at the end of this wash-out period (week −2 and day 0). Thereafter, the subjects were randomly assigned to receive either gemfibrozil (1200 mg/day) together with placebo matching Omacor™ capsules ($n = 16$) or Omacor™ capsules (4 g/day) together with placebo matching gemfibrozil for 12 weeks ($n = 14$) (day 0 to week 12). Blood samples were obtained at weeks 6, 10 and 12. The Omacor capsules (Pronova Biocare, Oslo, Norway) contained 1 g of concentrated n-3 FA (92%): 44.4% eicosapentaenoic (EPA) and 36.2% docosahexaenoic acid (DHA). D-alpha-tocopherol was added as an antioxidant to a concentration of 4 IU/g = 3.3 mg/g. The placebo capsule contained corn oil (56.3% linoleic acid), mono-unsaturated FA (26.8% oleic acid) and saturated FA (2.3% stearic acid), and 2.4 mg vitamin E.

For the evaluation of adverse events, serum enzyme activities (ALAT and ASAT), glucose and HbA_{1c} were determined according to the clinical routine at the hospital. Compliance was monitored by counting the returned capsules and was 98%.

2.3. Plasma

Venous blood samples were collected after an overnight fast into vacutainer tubes containing 1 mg/ml of ethylenediaminetetraacetic acid (K_3-EDTA). Plasma was isolated immediately and saccharose solution (final concentration 600 mg/ml H_2O) was added to prevent denaturation of lipoproteins during freezing; samples were stored at −80°C. All determinations were performed at the lipid research laboratory of the University Hospital Nijmegen.

2.4. Lipid and lipoprotein analysis

Very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) ($d < 1.019$ g/ml) were isolated by ultracentrifugation for 16 h at 36 000 rpm ($153\,000 \times g$) in a fixed-angle TFT 45.6 rotor (Kontron, Zurich). Plasma and lipoprotein cholesterol and triglyceride concentrati ns were determined with the Hitachi 744 analyser (cholesterol no 237574; triglyceride no 1361155; Boehringer-Mannheim, FRG). HDL cholesterol was determined in whole plasma using the phosphotungstate/Mg_{2+} method [25]. Apo E phenotypes were determined after iso-electric focusing of

VLDL lipoproteins, as described previously [26]. The apoE phenotypes were E4/3 ($n=10$), E3/3 ($n=6$), E3/2 ($n=12$), and E4/2 ($n=2$).

2.5. Analysis of low density lipoprotein subfraction profiles

LDL subfractions before and after treatment were separated by single spin density gradient ultracentrifugation [27]. Each individual LDL subfraction profile was defined by a continuous variable K, as described in detail previously [28,29]. Briefly, after ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Up to five LDL subfractions could be distinguished, i.e. LDL1 ($d=1.030$–1.033 g/ml), LDL2 ($d=1.033$–1.040 g/ml), LDL3 ($d=1.040$–1.045 g/ml), LDL4 ($d=1.045$–1.049 g/ml) and LDL5 ($d=1.049$–1.054 g/ml). The subfractions were carefully aspirated by means of a pasteur pipette. The volumes were calculated by weighing after correction for the densities. Subsequently, cholesterol was determined in each fraction; the concentrations were corrected for dilution and incomplete recoveries. The relative cholesterol concentrations (%chol) in the LDL subfractions were used to calculate parameter K as a continuous variable, which best describes each individual LDL subfraction profile. The relative contribution of each LDL subfraction, expressed by its cholesterol concentration (%chol LDL1–LDL5) relative to the total LDL subfraction profile (total LDL (100%) = %chol LDL1 + %chol LDL2 + %chol LDL3 + %chol LDL4 + %chol LDL5) was calculated. The relative cholesterol concentration of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give %chol LDL3′ = (%chol LDL3 + %chol LDL4 + %chol LDL5), where LDL [100%] = LDL1 (%chol LDL1) + LDL2 (%chol LDL2) + LDL3 (%chol LDL3′). When a subfraction pattern was characterized by a predominance of buoyant LDL particles, K was calculated by $K=$ (%chol LDL1 − %chol LDL3′)/(%chol LDL2 − %chol LDL3′) + 1). In the case of a predominancy of heavy, dense LDL particles, K was calculated by $K=$ (%chol LDL1 − %chol LDL3′)/(%chol LDL2 − %chol LDL1) + 1). A negative value ($K<0$) reflects a more dense LDL subfraction profile, and a positive K-value ($K>0$) a more buoyant profile.

2.6. Oxidation of low density lipoproteins

Plasma isolation was immediately followed by LDL isolation by density gradient ultracentrifugation (40 000 rpm for 18 h at 4°C) using a SW40 rotor (Beckman, Palo Alt , CA, USA). After isolation of total LDL, the protein content of LDL was measured by the method of Lowry et al. [30], with chloroform extraction to remove turbidity, using bovine serum albumin as a standard. LDL cholesterol was calculated by subtracting VLDL + IDL cholesterol and HDL cholesterol from total cholesterol. The oxidation experiments were performed as described by Esterbauer et al. [31], as modified by Kleinveld et al. [32]. Briefly, the oxidation of LDL (60 μg apolipoprotein/ml) was initiated by the addition of $CuSO_4$ to a final concentration of 18 μM at 37°C. The kinetics of the oxidation of LDL was determined by monitoring the change of the 234-nm diene absorption in a thermostated UV spectrophotometer. The oxidation characteristics of LDL were determined as described previously by the lag time (min), the oxidation rate (nmol dienes/mg protein per min) and the maximal amount of dienes formed during LDL oxidation (nmol/mg LDL protein) [33].

Thiobarbituric acid reactive substances (TBARS) in plasma were determined as described [34].

2.7. Determination of fatty acids and vitamin E in low density lipoprotein

Analysis of fatty acids, extracted from LDL by vortex mixing with 3 ml n-hexane, was performed by gas chromatography (Varian 3400 GC, Houten, The Netherlands) [33]. Vitamin E concentrations were determined by high-performance liquid chromatography (HPLC Spectra Physics Model 8800), with fluorescence detection. For extraction of vitamin E, 0.2 ml LDL was vortex mixed with 2 ml acetone and 2 ml petroleum ether [35].

2.8. Statistics

The values of the variables measured at week 0 and 12 are presented as the value 'before' and 'after' treatment, respectively. Results are expressed as mean ± S.D. and median with interquartile ranges. The mean of the individual percentage change after therapy was calculated and presented as delta (%).

The effect of either gemfibrozil or Omacor on absolute values of plasma lipoproteins, fatty acid composition, vitamin E concentration, TBARS and LDL oxidizability parameters were tested by non-parametric tests for dependent variables by the Wilcoxon signed rank test. Differences between the effects of gemfibrozil and Omacor on plasma lipoproteins, fatty acid composition, vitamin E concentration, TBARS and LDL oxidizability parameters were tested by the non-parametric Mann–Whitney U-tests for independent variables. A two-tailed probability value of less than 0.05 was considered to be significant. Pearson's correlation coefficients were computed to determine the correlation between the variables fatty acids and oxidazibility of LDL. The statistical analyses were performed with procedures available in the SPSS PC + (Statistical Package for the Social Sciences) software package Version 9.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Patients

Analysis was based on intention-to-treat, but two subjects were not included in the final analysis. One subject (Omacor group) developed excessive hypertriglyceridemia (TG = 56.5 mmol/l) after he stopped his regular medication. One subject (gemfibrozil group) was not willing to continue the trial after randomization.

At baseline, the gemfibrozil group ($n = 13$) and the Omacor group ($n = 15$) were similar in mean age and body mass index (BMI) (mean age 52.7 ± 6.9 vs 48.3 ± 8.3 years, respectively; BMI 26.6 ± 3.8 vs 27.5 ± 2.4 kg/m^2, respectively). After treatment, in the Omacor group eight patients showed an increase in body weight whereas in seven patients body weight remained stable or decreased. Similarly, in the gemfibrozil group six patients showed an increase in body weight whereas seven patients showed a stable or decreased body weight.

After inclusion, in both the gemfibrozil and Omacor group one patient with glucose levels above 6.9 mmol/l was present (gemfibrozil group, $n = 1$, glucose 10.0 and 10.1 mmol/l at week 0 and 12, respectively; Omacor group, $n = 1$, glucose 12.1 and 10.5 mmol/l at week 0 and 12, respectively). All other patients had glucose levels below 7.0 mmol/l. These two patients did not have a history of diabetes mellitus and were not treated with oral blood glucose lowering agents either before or during the trial. The fasting glucose concentrations as well as the hemoglobin A_{1c} concentration were similar at baseline levels for both drugs (gemfibrozil glucose 6.15 ± 2.04 mmol/l and HbA_{1c} 5.66 ± 0.67% vs Omacor glucose 5.88 ± 1.24 mmol/l and HbA_{1c} 5.54 ± 0.55%) and did not change during treatment with Omacor or gemfibrozil (data not shown). Omacor and gemfibrozil were tolerated well by all patients and no significant side-effects were observed.

3.2. The effect of treatment on plasma lipids and lipoprotein levels

The results for lipid and lipoprotein concentrations at baseline and after 12 weeks of treatment with gemfibrozil or Omacor are summarized in Table 1. Although baseline values of triglyceride and cholesterol levels of patients in the Omacor group are higher than in the gemfibrozil group this is not significant (P-value for triglyceride 0.44 and for cholester l 0.25 (Mann–Whitney U-test)).There were no significant differences between the two groups at baseline. Both gemfibrozil and Omacor significantly reduced total triglyceride levels in plasma as well as in the VLDL fraction. In addition, the VLDL cholesterol significantly decreased in both treatment groups. However, only a slight reduction in total cholesterol was found, reaching statistical significance in the Omacor group only, due to significant increase in HDL cholesterol and LDL cholesterol levels in both groups after treatment. There were no significant differences between the effects of gemfibrozil or Omacor on plasma lipoproteins.

3.3. The effect of treatment on LDL subfraction profile and K-value

The hypertriglyceridemic LDL before therapy tended to be polydisperse, consisting of multiple subfractions (LDL1–LDL5) over a broad density range (d = 1.030–1.054 g/ml), with the dense LDL subfractions (LDL3–LDL4) contributing most to total LDL (Fig. 1). This dense LDL subfraction profile is reflected by a negative value for parameter K, which did not differ significantly between the groups at baseline (Table 1). Both gemfibrozil and Omacor increased total LDL cholesterol (Table 1) by increasing the cholesterol content of LDL1, LDL2 and LDL3, whereas the relative contribution of LDL4 and LDL5 to total LDL decreased (Fig. 1), thus resulting in a more buoyant LDL subfraction profile. This is reflected by the increase in the value of parameter K after either gemfibrozil or Omacor therapy (Table 1). The value of parameter K increased more after gemfibrozil (+26.5%, $P < 0.01$) than after Omacor (+10.3%, $P = 0.05$) but the difference in change of parameter K between gemfibrozil and Omacor did not reach statistical significance ($P = 0.088$).

3.4. The effect of treatment on fatty acid composition and vitamin E content of LDL

In the gemfibrozil group the relative contribution of palmitic acid (16:0), and oleic acid (18:1) decreased significantly, whereas that of stearic acid (18:0), linolic acid (18:2), arachidonic acid (20:4), EPA (20:5) and DHA (22:6) did not change significantly (Table 2). In the Omacor group the relative contribution of EPA and DHA increased significantly, with a significant decrease of stearic acid and oleic acid, whereas the relative contribution of linoleic acid and arachidonic acid did not change (Table 2). Vitamin E in LDL increased significantly in both treatment groups. The total amount of polyunsaturated fatty acids (PUFA) in LDL tended to increase in both groups, just reaching statistical significance only in the gemfibrozil group. The ratio PUFA/vitamin E in LDL decreased significantly in both groups (Table 2). No significant difference between the effect of gemfibrozil and Omacor on fatty acid composition and vitamin E content of total LDL was found, except for palmitic acid and DHA and with borderline significance for EPA (Table 2).

3.5. The effect of treatment on oxidation of LDL

The lag time decreased significantly among the subjects treated with Omacor, whereas treatment with gemfibrozil did not affect the lag time (Table 3). A significant difference between the effect of gemfibrozil and Omacor on lag time was found ($P < 0.001$). Although the rate of oxidation tended to increase, the differences were not significant in any treatment group. Total amount of dienes produced per milligram of LDL protein increased in both groups after treatment, reaching statistical significance in the Omacor group and borderline significance in the gemfibrozil group (Table 3). No difference between the effect of gemfibrozil and Omacor on the rate of oxidation and amount of dienes was found (Table 3). TBARS concentrations in plasma increased after treatment with Omacor (+39%) and decreased after treatment with gemfibrozil (−6%); however, both changes failed to reach statistical significance. No significant difference in change in TBARS between both treatment groups was found (Table 3).

4. Discussion

The underlying cause of the increased tendency toward cardiovascular diseases in patients with hypertriglyceridemia is probably related to the enhanced atherogenic potential of their lipoproteins. Possible mechanisms contributing to this increased atherogenicity include the presence of small, dense LDL and the enhanced susceptibility to oxidative modification. In this report we described the baseline lipoprotein concentrations, the LDL subfraction profile and LDL oxidizability of patients with hypertriglyceridemia, and compared the effectiveness of treatment with either gemfibrozil or Omacor on these parameters in a double-blind, double-dummy design.

4.1. Lipids, lipoproteins and LDL heterogeneity

The observed reduction in plasma triglyceride, VLDL cholesterol and VLDL triglyceride concentrations and increase in HDL cholesterol concentrations

Table 1
Changes in lipid and lipoprotein concentration and the LDL subfraction profile (i.e. K-values) in subjects with hypertriglyceridemia after treatment with Omacor or gemfibrozil[a]

	Drug	Before	After	Delta (%)	P^b	P^c
Total cholesterol	O	8.85 ± 3.04 7.69 (6.15–11.87)	7.85 ± 2.32 7.16 (6.03–9.14)	−8.9 ± 14.8	<0.05	0.65
	G	7.15 ± 1.60 7.04 (5.76–8.95)	6.47 ± 1.16 6.26 (5.37–7.45)	−7.4 ± 15.3	0.06	
Triglycerides	O	9.79 ± 6.51 6.93 (6.00–11.26)	5.24 ± 2.80 4.53 (3.47–6.50)	−37.1 ± 25.5	<0.001	0.68
	G	6.99 ± 2.93 7.09 (4.63–8.02)	3.58 ± 2.27 2.92 (1.99–4.70)	−40.4 ± 52.6	0.01	
HDL cholesterol	O	0.71 ± 0.17 0.70 (0.56–0.84)	0.77 ± 0.18 0.73 (0.63–0.88)	+11.0 ± 18.5	<0.05	0.29
	G	0.79 ± 0.16 0.84 (0.65–0.93)	0.91 ± 0.19 0.81 (0.77–1.07)	+17.1 ± 21.4	<0.05	
VLDL cholesterol	O	5.17 ± 3.17 4.33 (2.72–9.13)	3.38 ± 2.37 2.53 (1.82–4.11)	−33.2 ± 22.3	<0.001	0.41
	G	3.23 ± 1.38 2.88 (2.36–4.33)	1.58 ± 0.78 1.45 (0.85–2.30)	−39.7 ± 55.2	<0.01	
VLDL triglycerides	O	8.76 ± 5.98 6.06 (5.38–10.54)	4.46 ± 2.31 4.01 (2.90–5.40)	−39.2 ± 26.7	<0.001	0.75
	G	6.22 ± 2.71 6.52 (3.80–7.52)	2.99 ± 2.19 2.27 (1.46–4.23)	−42.2 ± 60.1	0.01	
LDL cholesterol	O	2.97 ± 1.03 2.73 (2.31–3.06)	3.70 ± 1.00 3.65 (2.92–3.99)	+29.7 ± 31.2	0.005	1.00
	G	3.13 ± 0.87 3.65 (2.33–3.86)	3.98 ± 1.12 4.25 (3.28–4.93)	+33.6 ± 49.8	<0.05	
K-value	O	−0.61 ± 0.13 −0.62 (−0.71/−0.55)	−0.55 ± 0.16 −0.57 (−0.64/−0.52)	+10.3 ± 22.4	0.05	0.088
	G	−0.61 ± 0.11 −0.61 (−0.71/−0.52)	−0.45 ± 0.20 −0.41 (−0.60/−0.37)	+26.5 ± 33.6	<0.01	

[a] Values are presented in mmol/l, except parameter K. Results are expressed as mean ± S.D. and median with interquartile range. . gemfibrozil ($n = 13$); HDL, high density lipoprotein; LDL, low density lipoprotein; O, Omacor ($n = 15$); VLDL, very low density lipoprotein. 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.

[b] P-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.

[c] P-value for the between treatment groups Mann–Whitney U-test (gemfibrozil vs Omacor).

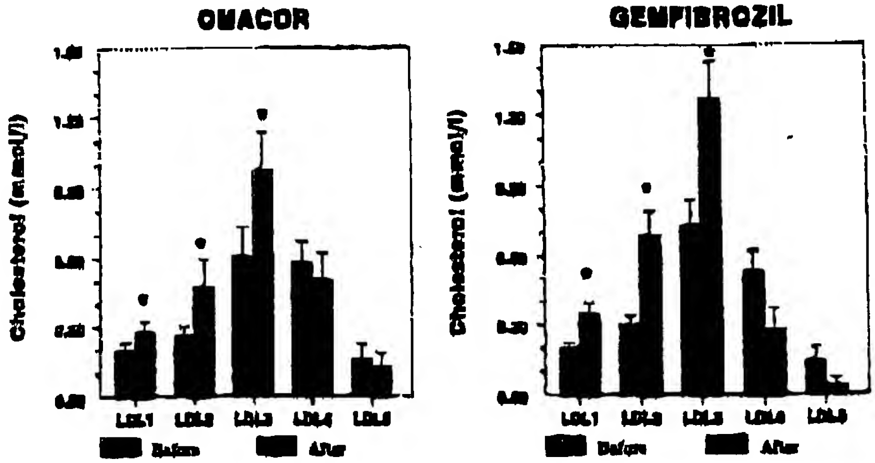

Fig. 1. Effect of treatment with either Omacor ($n = 15$) or Gemfibrozil ($n = 13$) on the cholesterol content of five LDL subfractions (LDL1–LDL5) of patients with hypertriglyceridemia: 'before' denotes values at week 0; 'after' denotes values at week 12. * P-value < 0.05 for the within treatment group, Wilcoxon signed ranks test (before vs after). No significant difference between the effect of Omacor and gemfibrozil treatment on the cholesterol concentration of the LDL subfractions was found.

with gemfibrozil and Omacor after 12 weeks of treatment (Table 1) are in accordance with previous reports [10–12,36]. Our knowledge of the possible mechanisms by which fibrates and eicosanoids induce these changes in lipid and lipoprotein concentrations has evolved greatly since the identification of the peroxisome proliferator-activated receptors (PPARs) [37]. Both eicosanoids and fibrates activate PPARs resulting in an enhanced catabolism of triglyceride-rich particles by decreased production of apoCIII and induction of LPL gene expression [38]. In addition, PPAR activation results in reduced secretion of VLDL particles by increased beta oxidation of fatty acids and inhibition of de-novo fatty acid synthesis [39]. The increase in LDL cholesterol by ~30% on both therapies is slightly larger than previously reported [10–12,36]. The depletion of triglycerides in the VLDL + IDL fraction induced by gemfibrozil and Omacor, leading to small, more dense VLDL + IDL particles which are more likely to be converted into LDL particles, has been suggested to be a cause of the observed increase in LDL cholesterol. So, in this study the high triglyceride levels at baseline may have contributed to the rather marked increase in LDL cholesterol concentrations [40].

The main LDL subfractions before therapy were abnormally small and dense (LDL3 and LDL4), resulting in a dense LDL subfraction profile, reflected by a negative value of parameter K. A dense LDL subfraction profile has been associated with an increased risk for CHD [6–8]. The predominance of small dense LDL in hypertriglyceridemia can be explained by exchange of LDL cholesteryl ester for VLDL triglyceride, mediated by cholesteryl ester transfer protein, followed by subsequent action of lipoprotein lipase or hepatic lipase, resulting in hydrolysis of LDL triglycerides and thereby decreasing LDL particle size [41]. Both gemfibrozil and Omacor treatment resulted in a more buoyant LDL subfraction profile, reflected by the increase in the value of parameter K (Fig. 1 and Table 1). So, Omacor and gemfibrozil adversely raise LDL cholesterol concentration but the increase in LDL cholesterol concentration reflects a less atherogenic light LDL subfraction profile that may be favorable. Similar results have been reported in hypertensive subjects [14] and in patients with familial combined hyperlipidemia (FCH) [15] after Omacor treatment and in hypertriglyceridemic subjects [4] and in patients with FCH [17] after clofibrate and gemfibrozil treatment, respectively. However, Omacor treatment in normolipidemic healthy subjects decreased LDL lipids and increased LDL apoB, thus decreasing the cholesterol/apoB ratio, reflecting more dense LDL, whereas no detectable differences in LDL size was found [16]. A possible explanation for this contrasting result is that the change in LDL composition depends on the extent of triglyceride transfer and lipolysis, determined by the degree of hypertriglyceridemia, which differed between the different reports.

Reportedly, alterations in composition of LDL particles were associated with changes in LDL metabolism in cultured cells, which may render them more atherogenic [2,3]. Another potential mechanism that increases the atherogenicity of LDL includes the oxidative modification [9].

4.2. LDL oxidizability

Oxidative modification of LDL involves the peroxidation of unsaturated fatty acids found within the LDL phospholipid monolayer. Several studies have shown that various types of fatty acids can alter LDL particle susceptibility to oxidative modification [42–44].

The results of studies on the effects f n-3 FA on LDL oxidizability are contradictory. In some of the studies enhanced peroxidation of LDL was observed [18–20], whereas other studies showed no effect of dietary n-3 FA on LDL oxidation [16,21,22]. Different experimental conditions among studies, e.g. in duration of supplementation period, type of patients included, amount of n-3 FA provided, may explain some of the apparently conflicting results obtained regarding the effects of n-3 FA on LDL oxidation.

We show that the lag time of LDL oxidation was significantly shortened by Omacor, which indicates an increase in the susceptibility to oxidation of LDL, as reported previously [18–20]. The trend of increase in TBARS concentration in plasma after treatment with Omacor corresponds with the increased susceptibility to oxidation of LDL in vitro. Several studies have demonstrated that small, dense LDL is more prone to oxidative modification in vitro than the large, light LDL, as measured by the decreased lag time, preceding the onset of the lipid peroxidation, suggesting an enhanced atherogenic potential of the small dense LDL subfrac-

Table 2
Change in fatty acid composition and vitamin E content of total LDL after treatment with Omacor or gemfibrozil in subjects with hypertriglyceridemia[a]

	Drug	Before	After	Delta (%)	p^b	p^c
Palmitic acid (C16:0)	O	23.3 ± 2.0 23.6 (20.8–25.0)	23.6 ± 2.2 23.8 (21.6–25.4)	+1.6 ± 6.6	0.39	0.022
	G	24.1 ± 2.0 23.6 (23.0–25.5)	23.1 ± 3.0 23.0 (22.0–23.6)	−4.0 ± 5.7	0.05	
Stearic acid (C18:0)	O	7.9 ± 0.7 7.9 (7.7–8.5)	7.5 ± 0.6 7.3 (6.8–8.2)	−5.1 ± 7.8	0.015	0.65
	G	7.5 ± 0.5 7.3 (7.2–7.7)	7.1 ± 0.7 7.0 (6.5–7.6)	−4.8 ± 11.0	0.14	
Oleic acid (C18:1 n-9)	O	19.5 ± 2.2 20.1 (17.6–21.0)	17.6 ± 1.6 17.3 (16.4–18.9)	−9.2 ± 8.4	0.003	0.47
	G	18.6 ± 2.8 19.6 (16.1–20.6)	17.7 ± 4.2 18.5 (14.3–19.3)	−5.0 ± 14.8	0.02	
Linoleic acid (C18:2 n-6)	O	40.8 ± 3.5 41.1 (37.9–41.5)	39.8 ± 5.3 39.5 (36.0–45.2)	−2.2 ± 10.7	0.31	0.16
	G	40.8 ± 5.3 41.3 (36.7–45.6)	42.1 ± 5.3 43.1 (36.7–47.8)	+3.3 ± 11.1	0.15	
Arachidonic acid (C20:4 n-6)	O	5.9 ± 1.2 5.9 (4.9–6.6)	5.8 ± 1.4 5.2 (5.0–6.5)	−1.0 ± 14.5	0.73	0.93
	G	7.0 ± 1.4 6.7 (6.0–8.2)	7.0 ± 1.6 7.4 (5.3–7.6)	+0.1 ± 15.3	0.92	
EPA (C20:5 n-3)	O	1.0 ± 0.5 1.1 (0.6–1.3)	3.1 ± 1.9 3.6 (0.8–4.9)	+231.0 ± 236.3	0.005	0.065
	G	0.9 ± 0.7 0.7 (0.5–1.5)	1.5 ± 1.4 0.8 (0.6–2.7)	+82.6 ± 183.6	0.22	
DHA (C22:6 n-3)	O	1.7 ± 0.5 1.7 (1.3–2.0)	2.6 ± 0.8 2.9 (1.8–3.1)	+68.7 ± 66.2	0.006	0.046
	G	1.3 ± 0.6 1.2 (0.8–1.7)	1.6 ± 0.8 1.5 (0.8–2.4)	+20.3 ± 58.4	0.42	
Vitamin E (μmol/g LDL protein)	O	6.53 ± 1.71 7.10 (4.60–7.90)	8.41 ± 1.97 8.30 (7.10–10.1)	+36.5 ± 50.0	<0.05	0.89
	G	6.95 ± 2.39 7.10 (5.05–8.50)	8.87 ± 3.11 8.40 (6.25–10.05)	+37.6 ± 59.4	<0.05	
Total amount of PUFA in LDL (μmol)	O	3596 ± 326 3513 (3323–3963)	3779 ± 535 3752 (3341–4251)	+5.0 ± 10.8	0.09	0.92
	G	3839 ± 271 3872 (3702–4048)	4008 ± 338 4000 (3758–4241)	+4.5 ± 7.0	<0.05	
PUFA/vitamin E in LDL (μmol/mg)	O	592 ± 183 523 (461–738)	468 ± 103 443 (372–550)	−17.4 ± 18.5	<0.001	0.79
	G	628 ± 261 529 (446–764)	499 ± 161 467 (407–652)	−14.8 ± 26.5	<0.001	

[a] Values f fatty acids are presented in percentage of total fatty acids as mean ± S.D. and median with interquartile ranges. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; G, gemfibrozil ($n = 13$); LDL, low density lipoprotein; O, Omacor ($n = 15$); PUFA, polyunsaturated fatty acids. 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.

[b] P-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.

[c] P-value for the between treatment groups Mann–Whitney U-test (gemfibrozil vs Omacor).

Table 3
Change in LDL oxidizability after treatment with Omacor or gemfibrozil[a]

	Drug	Before	After	Delta (%)	P^b	P^c
Lag time	O	85.7 ± 8.28 85.6 (80.5–92.0)	69.7 ± 8.2 68.0 (66.2–74.5)	−18.6 ± 7.6	<0.001	0.001
	G	74.6 ± 8.8 72.9 (69.5–81.2)	75.3 ± 10.0 72.7 (67.3–85.6)	+2.3 ± 13.8	0.70	
Oxidation rate	O	11.0 ± 1.83 10.6 (9.9–11.9)	11.2 ± 2.65 10.9 (8.1–13.2)	+2.0 ± 20.7	0.84	0.34
	G	11.9 ± 1.60 12.0 (11.1–12.9)	12.9 ± 2.63 12.8 (11.3–15.0)	+10.4 ± 23.4	0.14	
Dienes	O	468 ± 54 443 (431–494)	522 ± 90 521 (438–592)	+14.0 ± 17.0	0.01	0.62
	G	499 ± 46 498 (468–534)	541 ± 81 532 (507–603)	+9.9 ± 13.3	0.055	
TBARS	O	1.48 ± 0.74 1.19 (0.97–1.85)	1.88 ± 0.86 1.73 (1.17–2.54)	+38.7 ± 65.7	0.14	0.16
	G	1.24 ± 0.19 1.05 (0.93–1.58)	1.04 ± 0.19 1.06 (0.90–1.20)	−5.8 ± 36.2	0.34	

[a] Values are presented as mean ± S.D. and median with interquartile ranges; lag time in minutes; oxidation rate in nmol dienes/mg protein per min; dienes in nmol/mg LDL protein. G, gemfibrozil ($n = 13$); O, Omacor ($n = 15$); TBARS, thiobarbituric acid reactive substances (O: $n = 10$; G: $n = 9$). 'Before' are values at week 0. 'After' are values at week 12. Delta (%) is mean of the individual percentage change.
[b] P-value for the within treatment group Wilcoxon signed ranks test (before vs after) on absolute values.
[c] P-value for the between treatment groups Mann–Whitney U-test (gemfibrozil vs Omacor).

tions within each LDL subfraction profile [4,32]. In contrast, we now report that Omacor treatment is associated with a more buoyant LDL subfraction profile and an enhanced susceptibility to oxidation of total LDL. Since the ratio total PUFA per vitamin E in LDL decreased after Omacor, we may assume that the increased n-3 FA content most likely caused the increased susceptibility to oxidation, as the degree of unsaturation of fatty acids is one of the main determinants of the susceptiblity of the lipoproteins to oxidation.

After gemfibrozil treatment the lag time and TBARS concentration did not change significantly. To our knowledge only three reports have previously published the effect of fibrates on LDL oxidizability in humans [4,17,24]. In primary hypertriglyceridemic subjects, clofibrate treatment reduced the susceptibility of LDL to oxidation, as measured by a significant increase in lagtime in isolated LDL subfractions after therapy [4]. In subjects with familial combined hyperlipidemia gemfibrozil also tended to increase the resistence of total LDL to oxidation, as the lag time increased after therapy, although not reaching statistical significance [17]. In patients with hyperlipidemia type IIA and IIB, bezafibrate reduced the propensity of LDL to undergo lipid peroxidation in vitro [24]. The mechanism by which fibrates exhibit antioxidant potential is still unknown. One study reports that the p-hydroxy-metabolite I is involved through free radical scavenger activity [45]. Our present data show only little effect on LDL oxidizability after gemfibrozil treatment, less than expected on the basis of the more buoyant LDL subfraction profile induced by gemfibrozil. A possible explanation is that LDL oxidizability is determined in total LDL, which is the addition of maximal five LDL subfractions, so small changes might remain undetected.

The rate of LDL oxidation did not change in either group. The maximal amount of dienes formed per milligram of LDL protein during oxidation of LDL was significantly increased after Omacor therapy. This could be attributed to the increased number of oxidizable groups (= double bonds) in LDL due to Omacor supplementation. Indeed, a significant correlation between the PUFA content and diene production was found in the Omacor group ($r = 0.61$, $P < 0.01$).

5. Conclusion

Gemfibrozil and Omacor have anti-atherogenic properties, as both therapies reduce the atherogenic potential of the lipoproteins by decreasing the concentration of cholesterol-enriched VLDL and increasing HDL concentration. Although total plasma LDL cholesterol concentration increases, the atherogenic potential of LDL seems to be less, as judged by the presence of a more buoyant LDL subfraction profile. In contrast to gemfibrozil, Omacor increased the susceptibility of LDL to oxidation in vitro. Although this could be unfavorable, it does not necessarily mean that n-3 FA are atherogenic in vivo. In animal studies, the incorporation of n-3 FA into LDL particles tended to render them more susceptible to oxidation in vitro, but no increase

in atherosclerotic lesion development [46] or even an anti-atherogenic effect [47] in vivo was found. These findings might be explained by the presence in vivo of antioxidant mechanisms that can attenuate the increased potential of n-3 FA in LDL to undergo oxidative modification. Furthermore, n-3 FAs are reported to have a wide range of biological effects that may be related to protection against atherogenesis, i.e. reduction of platelet aggregation and vasoconstriction [48–52] and antiarrhythmic effects [53]. These mechanisms can to some degree offset the potential unfavorable effect of n-3 FA incorporation into LDL. Alternatively, supplementation of n-3 FA with anti-oxidants may help prevent the susceptibility of LDL to peroxidative modification.

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